1 **Effects of early life adversity on meningeal mast cells and proinflammatory gene expression in male**

2 **and female *Mus musculus***

3 Natalia Duque-Wilckens[1,2], Erika Sarno[1], Robby E. Teis[1], Frauke Stoelting[1], Sonia Khalid[1], Zakaria Dairi[1],

4 Alex Douma[1], Nidia Maradiaga[2], Kyan Thelen[2], A.J. Robison[1], and Adam J. Moeser[2,*]

5

6 [1]Department of Physiology, Michigan State University, East Lansing, MI, 48824

7 [2]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State

8 University, East Lansing, MI, 48824

9

10 [*]To whom correspondence should be addressed:

11 Gastrointestinal Stress Biology Laboratory

12 Department of Large Animal Clinical Sciences

13 College of Veterinary Medicine

14 Michigan State University

15 East Lansing, MI 48824

16 moeserad@cvm.msu.edu

17

18 **ABSTRACT**

19   Exposure to early life adversity (ELA) in the form of physical and/or psychological abuse or neglect

20   increases the risk of developing psychiatric and inflammatory disorders later in life. It has been

21   hypothesized that exposure to ELA results in persistent, low grade inflammation that leads to increased

22   disease susceptibility by amplifying the crosstalk between stress-processing brain networks and the

23   immune system, but the mechanisms remain largely unexplored. The meninges, a layer of three

24   overlapping membranes that surround the central nervous system (CNS)- duramater, arachnoid, and

25   piamater – possess unique features that allow them to play a key role in coordinating immune trafficking

26   between the brain and the peripheral immune system. These include a network of lymphatic vessels that

27   carry cerebrospinal fluid from the brain to the deep cervical lymph nodes, fenestrated blood vessels that

28   allow the passage of molecules from blood to the CNS, and a rich population of resident mast cells, master

29   regulators of the immune system. Using a mouse model of ELA consisting of neonatal maternal separation

30   plus early weaning (NMSEW), we sought to explore the effects of ELA on duramater mast cell histology

31   and expression of inflammatory markers in male and female C57Bl/6 mice. We found that mast cell

32   number, activation level, and relative expression of pseudopodia differ across duramater regions, and that

33   NMSEW exerts region-specific effects on mast cells in males and females. Using gene expression analyses,

34   we next found that NMSEW increases the expression of inflammatory markers in the duramater of

35   females but not males, and that this is prevented by pharmacological inhibition of mast cells with

36   ketotifen. Together, our results show that ELA drives sex-specific, long-lasting effects on the duramater

37   mast cell population and immune-related gene expression, suggesting that the long-lasting effects of ELA

38   on disease susceptibility could be partly mediated by meningeal function.

39

INTRODUCTION

Exposure to early life adversity (ELA), in the form of physical and/or psychological abuse or neglect, increases the risk of developing disorders at a multisystemic level later in life[1–4]: children with history of ELA show higher rates of psychiatric diseases such as unipolar depression and substance abuse[5,6], but also increased prevalence of gastrointestinal disorders[7,8], multiple sclerosis[9], migraines[10], cardiovascular disease[11], and metabolic syndrome[12],among others. Since these disorders share a mechanistic origin involving inflammation[13–18], it has been proposed that ELA-driven changes in the immune system are the root underlying susceptibility to these diseases. Indeed, children[19,20] and adults[21–23] with history of ELA display elevated levels of inflammatory markers. This, together with findings that ELA increases sensitivity of brain circuits involved in threat processing[24–26], prompted the hypothesis ELA drives persistent systemic inflammatory states by amplifying the crosstalk between stress-processing brain networks and the immune system[27,28].

A crucial interface between the brain and the peripheral immune system is the meninges, a layer of three overlapping membranes - duramater, arachnoid, and piamater - that surround the central nervous system (CNS). Although originally thought to serve mostly as a physical protection, there is growing evidence the meninges play an active role in coordinating immune trafficking throughout the CNS[29] through a variety of mechanisms. First, vessels within the duramater, unlike cerebral vessels, are fenestrated, and thus open to the passage of molecules present in the blood[30]. Second, the meninges hold vascular channels connecting the skull bone marrow with the brain surface, allowing myeloid cell migration into the brain[31]. Third, the duramater provides a network of lymphatic vessels that carry cerebrospinal fluid from the brain to the deep cervical lymph nodes[32–35], which permits cerebrospinal-injected immune cells to traffic from the brain to the periphery. Finally, the meninges contain a rich population of resident immune cells, among which are mast cells[36], master regulators of both innate and adaptive immune responses[37].

Mast cells are multifunctional innate immune cells characterized by their biphasic response to stimuli, which involves an early release of a wide range of preformed granule mediators (e.g. histamine, serotonin, proteases) followed by release of *de novo* synthesized mediators (e.g. cytokines). These mediators allow them to not only orchestrate innate and adaptive immune responses[37], but also modulate activity of vascular and lymphatic endothelial cells[38], nerves[39], and glia[40,41]. Meningeal mast cells, specifically, are key in maintaining blood brain barrier integrity[42,43] and recruiting T-cells and neutrophils[44]. Menigeal mast cells can also mediate the inflammatory responses associated with brain stroke[42,45] and the activation of the trigeminal pain pathway associated with migraines[46]. Importantly, mast cells can

72   rapidly respond to stress[47–49], and ELA can persistently modify their activity and distribution[50]. Together,

73   this suggests that meningeal mast cells are ideally positioned to play a key role in the brain-immune

74   crosstalk driving persistent inflammation associated with ELA, but, to our knowledge, whether ELA has

75   long-lasting effects on meningeal mast cell biology remains undetermined.

76   Here, we assessed the effects of ELA on duramater mast cell histology and expression of

77   inflammatory markers with and without exposure to adult stress in male and female C57Bl/6 mice. Our

78   analyses included mouse interleukin 6 (IL-6) and mouse tumor necrosis factor alpha (TNF-alfa), major

79   proinflammatory cytokines associated with ELA-related diseases[51,52]; mouse vascular endothelial growth

80   factor C (VEGF-C), critical for meningeal lymphangiogenesis[53] and recently shown to be actively involved

81   in brain immunosurveillance[54]; as well as mouse chymotryptic serine proteinase (CMA1), mouse histidine

82   decarboxylase (Hdc), and mouse tryptophan hydroxylase 1 (Tph1), which reflect different pathways of

83   mast cell activity[55–57]. We used the model of neonatal maternal separation plus early weaning (NMSEW).

84   Previous work has demonstrated that adult animals exposed to neonatal maternal separation show

85   elevated peripheral[58–61] and central[62–64] inflammatory markers, are more susceptible to develop

86   gastrointestinal[65,66], autoimmune[67] and cardiorespiratory[68,69] abnormalities, and show increased anxiety

87   and depressive-like behaviors[58,70,71], making this an ideal model to assess whether meningeal immunity

88   plays a role in ELA-induced persistent systemic inflammation. We added an early weaning component to

89   this protocol to increase the likelihood of inducing robust, long-lasting phenotypes[72]. Finally, to further

90   assess whether mast cell activity plays a role in ELA-induced changes in meningeal biology, we

91   administered the mast cell inhibitor Ketotifen fumarate (Ketotifen) before exposure to adult stress. We

92   hypothesized that NMSEW would increase activity of mast cells and expression of inflammatory markers

93   in the duramater after exposure to adult stress, and that this would be prevented by administration of

94   Ketotifen.

95

96   **MATERIALS AND METHODS**

97   **Animals.** All animal procedures were performed in accordance with the regulations of the Michigan State

98   University animal care committee. Founding C57Bl/6 breeders were obtained from the Jackson

99   Laboratory (Bar Harbor, Me). All animals were kept in cages enriched with Nestlet and Bed-r'Nest® at 20–

100   23°C under a 12 h light/dark cycle with ad libitum access to food and water and housed in same sex groups

101   of 3-5 individuals or in adult male-female pairs for breeding purposes (male was removed from the cage

102   before litters were born). For all experiments, newborn litters were randomly assigned to one of two

103   conditions: neonatal maternal separation + early weaning stress (NMSEW) or normally handled (NH). For

104   experiment 1, all animals from all litters were exposed to adult stress. For all gene expression analyses

105   (experiments 2,3 and 4), each litter was divided and randomly assigned to one of the three experiments

106   to minimize litter effects.

107

108   **Neonatal maternal separation plus early weaning (NMSEW).** During postnatal (PN) days 1-17, pups in

109   the NMSEW group were daily separated from their dams and placed in individual containers for 180

110   minutes, after which they were returned to their home cage with their dam and siblings. They were left

111   undisturbed between separation events. On day 17, NMSEW pups were weaned and housed in same sex

112   sibling groups. HydroGel™ was provided for the first two days after weaning to ensure proper hydration.

113   Pups in the NH groups were left undisturbed until postnatal day 28, when they were weaned. All animals

114   were ear punched for individual identification on weaning day. After weaning, mice from NMSEW and NH

115   groups were left undisturbed until they reached 8 weeks old (PN 56, adult) **(Fig. 2A,3A).**

116

117   **Adult Stress:** The adult stress consisted of a shortened version of subchronic variable stress[73,74]: for three

118   consecutive days, adult animals were singly housed and exposed for 10 min to either tail suspension test,

119   restraint stress, or foot shock stress (20 total 0.45 mA shocks at random). This milder version of stress was

120   chosen to minimize ceiling effects based on evidence showing that early life adversity increases sensitivity

121   to future stress[75]. **In experiments 1 and 3** (Figs.2A,3A and 5A), all animals from both the NMSEW and NH

122   groups were exposed to adult stress on days 56-59. **In experiment 2** (Fig.4A), animals from the NMSEW

123   and NH groups were left undisturbed until tissue collection. **In experiment 4** (fig.6A), animals from both

124   the NMSEW and NH groups received an intraperitoneal injection of Ketotifen fumarate (see below) before

125   each episode of adult stress on days 56-59.

126

127   **Mast cell stabilization:** 20mg/kg Ketotifen (Cayman Chemical, Catalog #20303) was administered

128   intraperitoneally 30 min prior each episode of adult stress (fig.5A). Ketotifen fumarate is a histamine H1

129   receptor antagonist and mast cell stabilizer (prevents mast cell degranulation)[76,77] that crosses the blood

130   brain barrier[78]. The chemical structure of ketotifen fumarate is available in PubChem, PubChem CID:

131   5282408. 20 mg/kg dose was based on previously published studies showing successful mast cell

132   stabilization in mice[79–81].

133

134   **Tissue collection:** 3 days after the last episode of stress, animals were euthanized via cervical dislocation.

135   **In experiment 1**, the skull caps with attached duramater were collected and immediately placed into 10%

136   formalin solution. **In experiments 2, 3, and 4**, the duramater was freshly separated from the skull and

137   immediately flash frozen using dry ice.

138

139   **Duramater mast cell staining and quantification (experiment 1):** After 24h of postfixation with 10%

140   formalin, duramater was carefully separated from the skull, washed with DI water, and mounted on

141   Superfrost plus microscope slides (Fisher Scientific). Once dry, slides were immersed for 1h in a solution

142   containing 0.5% Toloudine blue in 0.5N Hydrochloric acid, after which they were washed in DI water,

143   dried, and coverslipped using Vectashield mounting medium. A Leica DM750 bright field Microscope with

144   ICC50W Integrated Camera was used to take four 10x brightfield pictures per each duramater region

145   available (fig.2A,3A): parietal, interparietal, sagittal sinus, and transverse sinus. Finally, quantification was

146   made by a blind experimenter using ImageJ. A 1000x780 μm box was used to limit counting areas:two

147   random and non-overlapping counting areas were used per picture, 8 counting areas total per duramater

148   region. The boxes for parietal and interparietal regions were placed parallel to the sinuses, so that the

149   sinus blood vessel and lumen were not included in the counting region. The total number of mast cells,

150   number of active mast cells, number of inactive mast cells, and number of mast cells expressing

151   pseudopodia were quantified per counting area. For detailed description of criteria used to classify mast

152   cells please refer to table 1. The level of activation was calculated as the number of active mast

153   cells/number of inactive mast cells, and the relative expression of pseudopodia was calculated as number

154   of mast cells expressing pseudopodia/total number of mast cells per counting area. The data are

155   presented as average numbers per area/mouse (total counts/number of counting areas) **(fig.2B,3B).**

156

157   **Gene expression analyses (experiments 2, 3, and 4):** Duramater was homogenized using Trizol

158   (Invitrogen), and total RNA was extracted using the RNAeasy MicroKit-Qiagen #74004. RNA integrity and

159   concentration were assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto,

160   California). Samples with RIN values equal to or above 8 were converted to cDNA using the Applied

161   Biosystems™ High-Capacity cDNA Reverse Transcription Kit (#4368814). The cDNA was used on the real-

162   time RT^2 Profiler PCR Array (QIAGEN, Cat. no. CLAM38697) in combination with RT^2 SYBR®Green qPCR

163   Mastermix (Cat. no. 330503). Each array plate contained one set of 96 wells with a panel of Qiagen

164   designed primers for mouse IL-6, TNF-alfa, Hdc, Tph1, VEGF-C, and CMA1. Positive PCR controls were

165   included in each 96-well set on each plate. Glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was

166   used as the assay reference gene. Each array contained samples from control and test groups.

167   **Statistical analyses:** All figures and statistical analyses were done using Graphpad Prism 9 software. For

168   all analyses, we used an alpha level criterion of 0.05 for statistical significance. One-way ANOVAs followed

169   by Dunnett's multiple comparison tests were used to compare mast cells between duramater regions (fig

170   1). Two-way ANOVAs were used to analyze effects of sex, early life treatment, and the interaction in all

171   experiments (figs.2-6), followed by Šídák's multiple comparisons test in ANOVAS showing significant

172   interaction effects.

173

174   **RESULTS**

175   **Mast cells are not homogeneous across duramater regions.**

176   Since the cellular compartment of the duramater is not homogeneous[29], we first assessed

177   whether there were overall differences in mast cell number, activation levels, and pseudopodia

178   expression among the major regions of the cortical duramater: interparietal, parietal, sagittal sinus, and

179   transverse sinus. We found that the region of the duramater had a significant effect on all the measures.

180   There was a main effect of region on the average of mast cells per mm^2 (one way ANOVA $F_{3,198}=185.5$,

181    $p<0.0001$, Fig. 1B), with the interparietal duramater showing significantly higher mast cell density than

182   any other region (Dunnett's multiple comparisons test interparietal vs. parietal $p<0.0001$, interparietal vs

183   sagittal $p<0.0001$, interparietal vs. transverse sinus $p<0.0001$). Similarly, there was a significant effect of

184   duramater region on activation level (one way ANOVA $F_{3,198}=34.96$, $p<0.0001$, Fig. 1C), with transverse

185   sinus showing the highest ratio of active to inactive mast cells (Dunnett's multiple comparisons test

186   transverse vs. parietal $p<0.0001$, transverse vs interparietal $p<0.0001$, transverse vs. sagittal sinus $p<0.01$).

187   Finally, the relative expression of pseudopodia, cytoplasmic extensions that mast cells use to transfer

188   granule content to neighboring cells[82] and/or engulf pathogenic cells[83] was also different between

189   duramater regions (one way ANOVA $F_{3,198}=138.8$, $p<0.0001$, Fig. 1D), with the interparietal duramater

190   showing the highest relative expression of pseudopodia (Dunnett's multiple comparisons test

191   interparietal vs. parietal $p<0.0001$, interparietal vs sagittal $p<0.0001$, interparietal vs. transverse sinus

192    $p<0.0001$).

193

194   **NMSEW induces changes in duramater mast cells in a region-specific fashion.**

195   Because our results demonstrate heterogeneous characteristics of meningeal mast cells
196   depending on the region of the duramater, all subsequent comparisons between treatments were done
197   within specific duramater regions. In the **interparietal** region of the duramater, early life treatment
198   affected total mast cell number and relative pseudopodia expression. Compared to NH animals, NMSEW
199   animals showed significantly higher mast cell number (two way ANOVA main effect of early life treatment
200    $F(1,48)=5.1$, $p=0.03$, **fig. 2D**) and lower relative expression of pseudopodia (two way ANOVA main effect
201   of early life treatment $F(1,48)=8.2$, $p=0.006$, **fig. 2F**). No differences were seen between groups in the
202   activation levels.

203   In the **parietal** duramater, there were no differences between groups in the mast cell number or
204   pseudopodia expression, but there was an interaction between early life treatment and sex in the
205   activation levels (two way ANOVA interaction $F(1,47)=7.9$, $p=0.007$): in males but not females, NMSEW
206   reduced the activation level (Šídák's multiple comparisons test $p=0.01$, **fig. 2I**).

207   In the **sagittal sinus** region, the treatment during early life also affected the relative expression of
208   pseudopodia, but, in contrast to the parietal region, NMSEW animals showed higher pseudopodia
209   expression (two way ANOVA main effect of early life treatment $F(1,48)=7.5$, $p=0.008$, **fig. 3F**). There were
210   no differences between groups in the total number or activation levels of mast cells.

211   Finally, in the **transverse sinus** region, while no effects of early life treatment were found in any
212   of the measures, there was an effect of sex on activation levels, with females showing higher activation
213   levels than males (two way ANOVA main effect of sex $F(1,45)=5.74$, $p=0.02$, **fig. 3I**).

214

215   **NMSEW increases duramater expression of proinflammatory molecules in females but not males.**

216   There was a significant interaction between early life treatment and sex in expression of CMA1
217   (two way ANOVA interaction effect $F(1,17)=5.008$, $p=0.04$, **fig. 4B**), IL-6 (two way ANOVA interaction effect
218    $F(1,17)=11.2$, $p=0.003$, **fig. 4C**), and TNF alpha (two way ANOVA interaction effect $F(1,17)=6.76$, $p=0.02$,
219   fig. **4E**). Šídák's multiple comparisons test revealed that NMSEW increased the expression of all three
220   genes in females (CMA1 $p=0.002$, IL-6 $p<0.0001$, TNF alpha $p=0.03$) but not in males. The expression of
221   VEGF-C was increased in animals exposed to NMSEW compared to NH (two way ANOVA main effect of
222   early life treatment $F(1,17)=6$, $p=0.026$, **fig. 4G**). We found no differences in the expression of Hdc or
223   Tph1 between groups.

224

225   **Exposure to a second hit stress doesn't interfere with the effects of NMSEW on meningeal gene**
226   **expression.**

227   Similar to experiment 2, in experiment 3 there was a significant interaction between early life

228   treatment and sex in expression of CMA1 (two way ANOVA interaction effect $F_{(1,13)}=13.34$, p=0.003, **fig.**

229   **5B**), IL-6 (two way ANOVA interaction effect $F_{(1,13)}=1.48$, p=0.04, **fig. 5C**), and TNF alpha (two way ANOVA

230   interaction effect $F_{(1,13)}=7.7$, p=0.02, **fig. 5E**). Šídák's multiple comparisons test revealed that NMSEW

231   increased the expression of CMA1 (p=0.007) and IL-6 (p=0.049) in females. NMSEW reduced the

232   expression of TNF alpha in males (p=0.034), while the effect in females was not significant. There was

233   also a significant interaction in the expression of VEGF-C (two way ANOVA interaction effect $F_{(1,13)}=5.4$,

234   p=0.036, **fig. 5G**), but no significant differences between groups were apparent after Šídák's multiple

235   comparisons test. No effects of early life treatment or sex were found in the expression of Hdc or Tph1.

236

237   **Stabilizing mast cells before exposure to adult stress eliminates the effects of NMSEW on meningeal**

238   **gene expression.**

239   In animals receiving Ketotifen, no effects of early life treatment or sex were seen in the expression

240   of any of the genes measured (fig. **6 B,C,D,E,F,G**). These data indicate that mast cell activity is critical for

241   the effects of NMSEW on meningeal gene expression.

242

243   **DISCUSSION**

244

245   In the present study we show that mast cell number, activation level, and relative expression of

246   pseudopodia differ across duramater regions, and that NMSEW exerts meningeal region-specific effects

247   on mast cells in males and females. Using gene expression analyses, we next showed that NMSEW

248   increases the expression of inflammatory markers in the duramater of females but not males, and that

249   this is prevented by pharmacological inhibition of mast cells with ketotifen. Together, our results show

250   that early life adversity results in sex-specific, long-lasting effects on meningeal mast cell population and

251   immune-related gene expression and suggest that that these are at least partly driven by changes in mast

252   cell activity.

253

254   *Early life adversity exerts long-term effects on duramater mast cells in a region-specific fashion.*

255   Using T-blue staining in whole mount duramater from adult animals, we found that mast cell

256   populations are not homogeneous across duramater regions. Compared to parietal, sagittal sinus, and

257   transverse sinus, the interparietal region showed the highest number of mast cells per mm^2 and the

258   highest relative expression of pseudopodia. Conversely, the transverse sinus showed the highest

259   activation levels. Based on these results and previous evidence showing that the duramater has location-

260   specific mechanical and biochemical properties[84], innervation[85,86], presence of blood and lymphatic

261   vessels[32,33], as well as cellular immunity[87], we separated the mast cell histology analyses by duramater

262   region.

263   In the **interparietal region** of the duramater, NMSEW increased the total mast cell number while

264   decreasing the relative activation levels, regardless of sex. This region of the duramater overlays the

265   cerebellum, a brain region involved in motor coordination but also in cognitive and emotional processes

266   [88–90]. Interestingly, human and animal studies have shown that ELA has long-term structural and functional

267   consequences on the cerebellum[91]. For example, physical/psychological neglect during early life can affect

268   cerebellar volume in healthy children[92] and adults suffering from obsessive compulsive disorder[93]. In rats,

269   NMSEW is associated with reduced cerebellar metabolic activity[94]. Although it is unknown whether these

270   effects of ELA on cerebellum are mediated by interparietal meninges, some evidence suggest that this

271   could be possible: the interparietal meninges are directly involved in the migration of cells from the

272   external germinal layer into the internal granule layer in the cerebellum during both embryonic and

273   postpartum period[95,96]. Future studies assessing the role of interparietal mast cells in cerebellar function,

274   and whether this is affected acutely and/or chronically by ELA, could reveal a novel communication

275   pathway between the meninges and the brain.

276   In the **sagittal sinus region**, NMSEW increased the relative expression of mast cell pseudopodia

277   regardless of sex. Pseudopodia represent a mechanism by which mast cells directly communicate with

278   adjacent cells in a process called transgranulation, in which mast cells directly transfer their granular

279   content to other cells[82,97]. The meningeal lymphatic network runs along the sagittal and transverse sinus

280   regions [32,33]. This network is critical for the cross-talk between the CNS and the peripheral immune system,

281   as it provides an exclusive route for draining macromolecules and trafficking immune cells from the

282   cerebrospinal fluid to the cervical lymph nodes[32,98,99]. Interestingly, NMSEW also increased the expression

283   of VEGF-C, the key growth factor for lymphatic vessel formation[53], and VEGF-C is actively involved in brain

284   immunosurveillance[54]. An elevated expression of VEGF-C has been associated with increased lymphatic

285   formation and/or flow[54,100]. Therefore, it is possible that, through the release of vascular mediators, mast

286   cells directly modulate meningeal lymphatic vasculature function[101]. The sagittal sinus is also an area

287   where CNS-derived antigens accumulate and are presented to patrolling T-cells[87]. There is substantial *in*

288   *vivo* and *in vitro* evidence demonstrating that mast cells can modulate T-cells' activity[102,103], and a recent

289   study showed specifically that meningeal mast cells play a crucial role in T-cell recruitment and induction

290   of pathogenesis in a model of experimental encephalomyelitis[104]. Taken together, an increased expression

291   of pseudopodia in the sagittal sinus could indicate an enhanced interaction between mast cells and

292   lymphatic vessels and/or T-cells, facilitating immune trafficking.

293

294   *Early life adversity exerts sex-specific effects on meningeal inflammatory markers.*
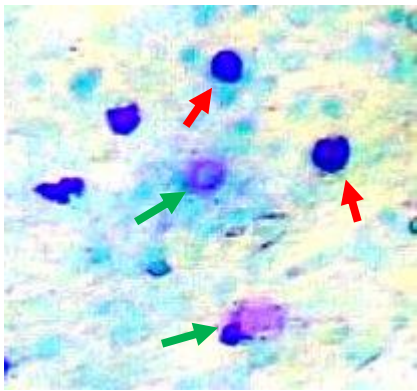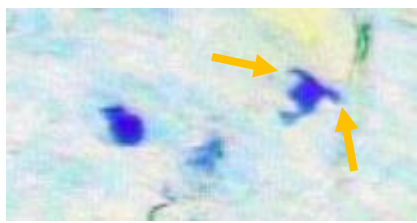
295   To assess whether ELA has long-term effects on meningeal immunology, we performed gene

296   expression analysis in whole duramater samples of adult males and females exposed to NMSEW. We

297   found that, while Tph1 and Hdc were not affected by early life treatment, CMA1, IL-6, and TNFαwere

298   elevated in females but not males exposed to NMSEW. These results suggest that females may exhibt

299   greater ELA-induced meningeal inflammation, which is in line with a wealth of research demonstrating

300   that women show stronger inflammatory responses[105–107] and heightened susceptibility to diseases

301   associated with ELA including anxiety and depression[108], migraines[109,110], and autoimmune disorders[111].

302   The present study provides evidence that this exaggerated NMSEW-induced dural inflammation in

303   females is mediated, at least in part, by mast cell activity. First, we found that inhibition of mast cell

304   function by administration of ketotifen was sufficient to prevent the proinflammatory effects of ELA.

305   Second, our histological analyses showed that, compared to males, females have elevated activity of mast

306   cells located in the dural transverse sinus, a key neuroimmune interface[87] as discussed above. Previous

307   work showed that mast cells from female mice have an increased baseline mediator storage capacity and

308   show exaggerated mediator release in response to psychological stress compared to mast cells from

309   males[112,113]. Since ELA exerts long term effects on mast cell function[50,114], it is possible that a female-biased

310   mast cell reactivity results in stronger proinflammatory effects. Whether there is a causal relationship

311   between increased meningeal inflammation and persistent, low-grade systemic inflammation contributes

312   to ELA-related disease risk remains to be elucidated.

313   In sum, our studies show that ELA can have long-lasting, sex-specific effects on meningeal function

314   and suggest that mast cells could play a critical role connecting the central nervous system with peripheral

315   immune responses. Since meninges are a crucial interface between the brain and peripheral immunity,

316   these results raise the intriguing hypothesis that meningeal mast cells could be directly contributing to

317   the enhanced crosstalk between stress-processingbrain networks and the immune system thought to

318   underly ELA-induced vulnerability to CNS disease[27,28,87]. A better understanding of the role of meningeal

319   mast cells in CNS and systemic inflammation could provide key molecular insights aiding development of

320   novel, mast cell-targeting therapeutics for stress-related diseases.

321   Acknowledgements

Table 1. Classification of duramater mast cells

Type	Description	Examples
Inactive (red arrows)	Mast cell is densely packed, most of the cytoplasm is dark purple, and the cell surface can be clearly delimited	
Active (green arrows)	Mast cell is not densely packed, the cytoplasm purple stain is scattered, and the cell surface is blurry	
Pseudopodia (yellow arrows)	One or more cytoplasmic extensions arise from the surface of the cell.	

Fig. 1. Mast cells are not homogeneous across duramater regions. A: diagram showing regions of duramater analyzed:: parietal, interparietal, Sagittal sinus and Transverse sinus. B: Total mast cell (MC) number per mm^2 significantly differs by duramater region, with interparietal region showing the highest mast cell density. C: Activation level of mast cells differs by duramater region, with transverse region showing the highest activation. D: Relative expression of pseudopodia varies by duramater region, with interparietal region showing the highest expression. One way ANOVA followed by Dunnett's multiple comparisons test, **$p < 0.01$, ****$p < 0.0001$. n=49-52 per group.
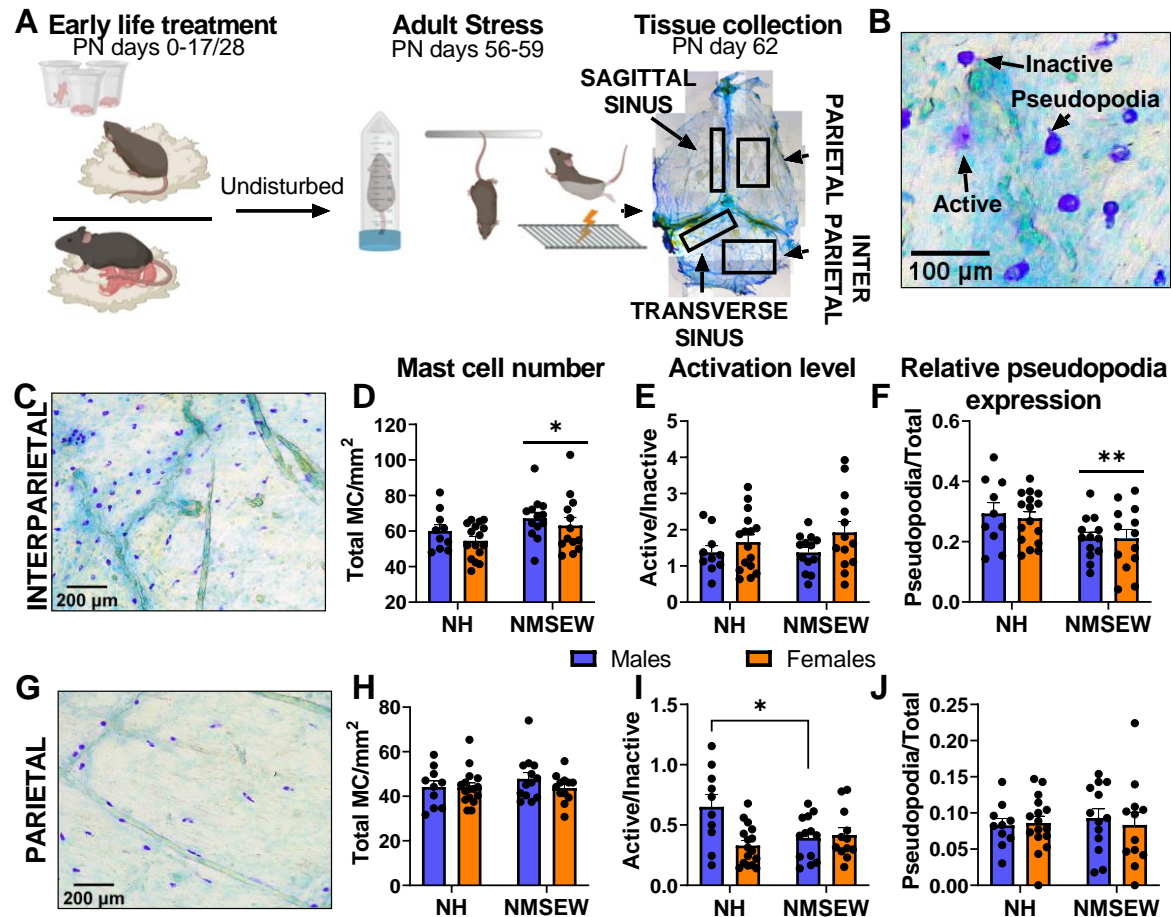
Fig. 2. Early life adversity exerts long-term effects on duramater mast cells in interparietal and parietal regions of the duramater. A: diagram showing experimental timeline. **B.** Photomicrograph showing examples of mast cell features quantified: active mast cell, inactive mast cell, and presence of pseudopodia. **C, G.** Photomicrograph showing examples of mast cells in interparietal and parietal regions, respectively. **D.** Compared to NH, NMSEW animals show higher numbers of mast cells in interparietal region (two way ANOVA main effect of early life treatment, *p=0.03). **F.** Compared to NH, NMSEW animals show lower relative expression of pseudopodia (two way ANOVA main effect of early life treatment **p=0.006). **I.** In the parietal region, NMSEW reduced the activation levels in males (Šídák's multiple comparisons test*p=0.01). No differences between groups were seen in **E.** interparietal activation levels of mast cells, **H.** parietal total mast cell number, and **J.** Parietal relative expression of pseudopodia. N= 10-16 per group.
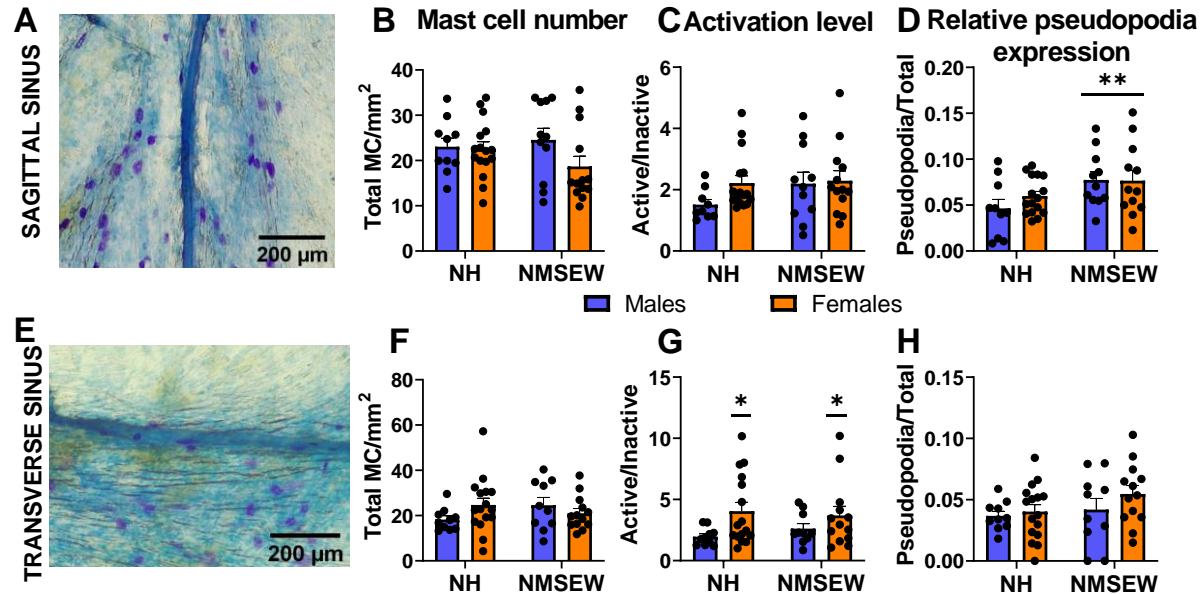
Fig. 3. Early life adversity exerts long-term effects on duramater mast cells in sagittal and transverse sinus regions of the duramater. A, B. Photomicrograph showing examples of mast cells in sagittal and transverse regions, respectively. **D.** Compared to NH, NMSEW animals show higher numbers of relative pseudopodia in the sagittal sinus region (two way ANOVA main effect of early life treatment, **p=0.008). **G.** In the transverse sinus region, females showed higher activation levels than males (two way ANOVA main effect of sex, *p=0.02). No differences between groups were seen in **B,F.** Mast cell number in sagittal or transverse sinus regions, **C.** Mast cell activation level in sagittal sinus region, or **H.** relative expression of pseudopodia in transverse sinus region. N= 10-16 per group.
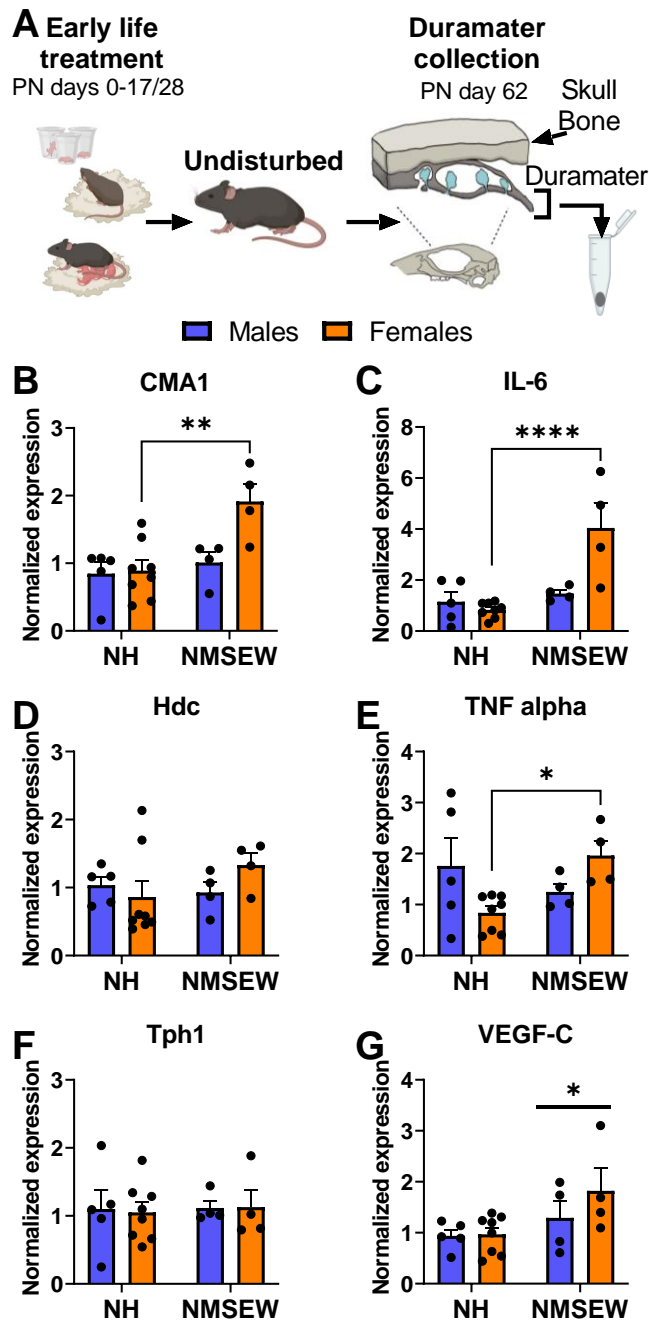
Fig. 4. NMSEW increases duramater expression of proinflammatory molecules in females but not males.
A. Experimental timeline. **B, C, E.** NMSEW increased expression of CMA1 (Šídák's multiple comparisons test **p=0.002) IL-6 (Šídák's multiple comparisons test ****p<0.0001), and TNF alpha (Šídák's multiple comparisons test *p=0.03) in females but not males **G**. The expression of VEGF-C was increased in animals exposed to NMSEW compared to NH (two way ANOVA main effect of early life treatment *p=0.026). No effects of sex or early life treatment where seen in **D**. Hdc, or **F**. Tph1. N= 4-8 per group.
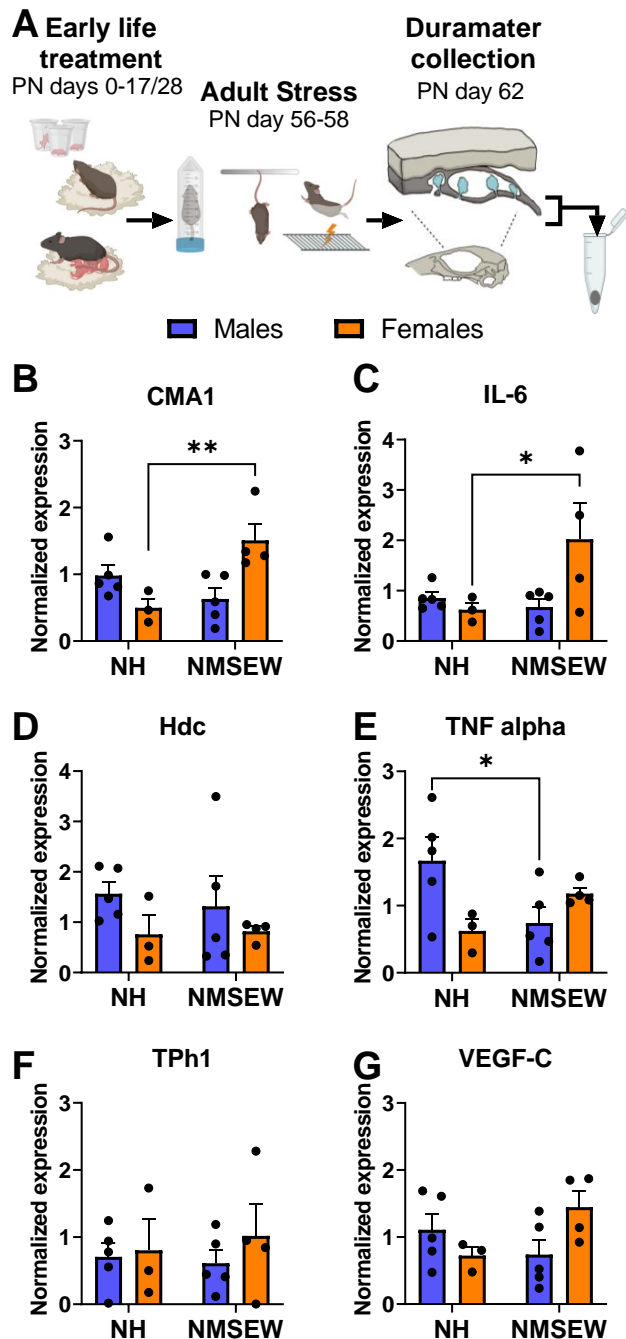
Fig. 5. **Exposure to a second hit stress doesn't interfere with the effects of NMSEW on meningeal gene expression. A.** Experimental timeline. **B, C.** NMSEW increased expression of CMA1 (Šídák's multiple comparisons test **p=0.007) and IL-6 (Šídák's multiple comparisons test *p=0.049) in females but not males. **E.** NMSEW reduced the expression of TNF alpha in males (Šídák's multiple comparisons test *p=0.034). No effects of sex or early life treatment where seen in **D**. Hdc, **F**. Tph1, of **G.** VEGF-C. N= 3-5 per group
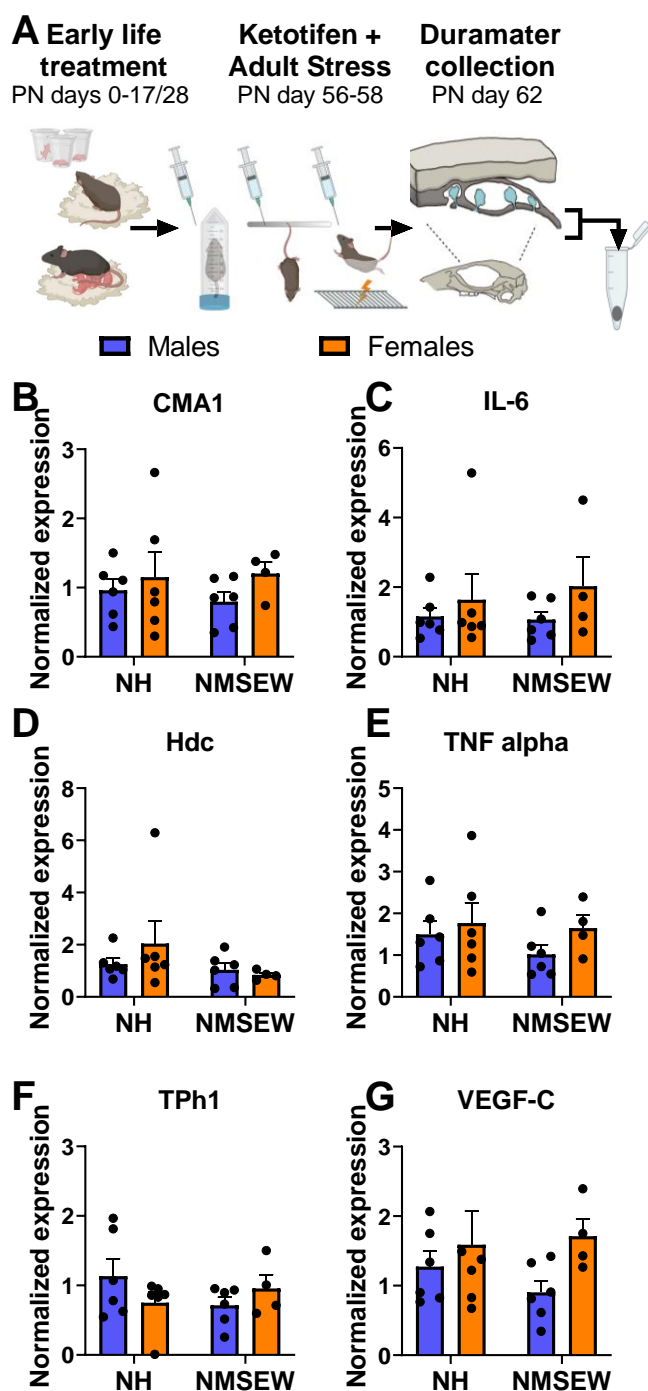
Fig. 6. **Stabilizing mast cells before exposure to adult stress eliminates the effects of NMSEW on meningeal gene expression. A.** Experimental timeline. B,C,D,E,F,G. No effects of early life treatment or sex were seen in the expression of any of the genes measured after administration of Ketotifen.

References

1. Varese, F. *et al.* Childhood adversities increase the risk of psychosis: a meta-analysis of patient-control, prospective- and cross-sectional cohort studies. *Schizophr. Bull.* **38**, 661–671 (2012).

2. Edwards, V. J., Holden, G. W., Felitti, V. J. & Anda, R. F. Relationship between multiple forms of childhood maltreatment and adult mental health in community respondents: results from the adverse childhood experiences study. *Am. J. Psychiatry* **160**, 1453–1460 (2003).

3. Goodwin, R. D. & Stein, M. B. Association between childhood trauma and physical disorders among adults in the United States. *Psychol. Med.* **34**, 509–520 (2004).

4. T. Jones, G. Psychosocial Vulnerability and Early Life Adversity as Risk Factors for Central Sensitivity Syndromes. *Curr. Rheumatol. Rev.* **12**, 140–153 (2016).

5. Kessler, R. C., Davis, C. G. & Kendler, K. S. Childhood adversity and adult psychiatric disorder in the US National Comorbidity Survey. *Psychol. Med.* **27**, 1101–1119 (1997).

6. McLaughlin, K. A. *et al.* Childhood Adversities and First Onset of Psychiatric Disorders in a National Sample of US Adolescents. *Arch. Gen. Psychiatry* **69**, 1151–1160 (2012).

7. Talley, N. J., Fett, S. L., Zinsmeister, A. R. & Melton, L. J. Gastrointestinal tract symptoms and self-reported abuse: a population-based study. *Gastroenterology* **107**, 1040–1049 (1994).

8. Bradford, K. *et al.* Association between early adverse life events and irritable bowel syndrome. *Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc.* **10**, 385-390.e1–3 (2012).

9. Spitzer, C. *et al.* Childhood Trauma in Multiple Sclerosis: A Case-Control Study. *Psychosom. Med.* **74**, 312–318 (2012).

10. Brennenstuhl, S. & Fuller-Thomson, E. The Painful Legacy of Childhood Violence: Migraine Headaches Among Adult Survivors of Adverse Childhood Experiences. *Headache J. Head Face Pain* **55**, 973–983 (2015).

11. Godoy, L. C. *et al.* Association of Adverse Childhood Experiences With Cardiovascular Disease Later in Life: A Review. *JAMA Cardiol.* **6**, 228–235 (2021).

12. Rich-Edwards, J. W. *et al.* Abuse in childhood and adolescence as a predictor of type 2 diabetes in adult women. *Am. J. Prev. Med.* **39**, 529–536 (2010).

13. Levy, D. Migraine pain, meningeal inflammation, and mast cells. *Curr. Pain Headache Rep.* **13**, 237–240 (2009).

14. Lee, C.-H. & Giuliani, F. The Role of Inflammation in Depression and Fatigue. *Front. Immunol.* **10**, (2019).

15. Martinez, P., Lien, L., Zemore, S., Bramness, J. G. & Sudan, P. N. Circulating cytokine levels are associated with symptoms of depression and anxiety among people with alcohol and drug use disorders. *J. Neuroimmunol.* **318**, 80–86 (2018).

16. Steven, S. *et al.* Vascular Inflammation and Oxidative Stress: Major Triggers for Cardiovascular Disease. *Oxid. Med. Cell. Longev.* **2019**, (2019).

17. Kaunzner, U. W. *et al.* Quantitative susceptibility mapping identifies inflammation in a subset of chronic multiple sclerosis lesions. *Brain* **142**, 133–145 (2019).

18. Monteiro, R. & Azevedo, I. Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm.* **2010**, (2010).

19. Slopen, N., Kubzansky, L. D., McLaughlin, K. A. & Koenen, K. C. Childhood adversity and inflammatory processes in youth: A prospective study. *Psychoneuroendocrinology* **38**, 188–200 (2013).

20. Danese, A. *et al.* Biological embedding of stress through inflammation processes in childhood. *Mol. Psychiatry* **16**, 244–246 (2011).

21. Danese, A., Pariante, C. M., Caspi, A., Taylor, A. & Poulton, R. Childhood maltreatment predicts adult inflammation in a life-course study. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 1319–1324 (2007).

22. Kiecolt-Glaser, J. K. *et al.* Childhood Adversity Heightens the Impact of Later-Life Caregiving Stress on Telomere Length and Inflammation. *Psychosom. Med.* **73**, 16 (2011).

23. Loucks, E. B. *et al.* Life course socioeconomic position is associated with inflammatory markers: The Framingham Offspring Study. *Soc. Sci. Med.* **71**, 187–195 (2010).

24. Tottenham, N. *et al.* Elevated amygdala response to faces following early deprivation. *Dev. Sci.* **14**, 190–204 (2011).

25. van Harmelen, A.-L. *et al.* Enhanced amygdala reactivity to emotional faces in adults reporting childhood emotional maltreatment. *Soc. Cogn. Affect. Neurosci.* **8**, 362–369 (2013).

26. Kim, P. *et al.* Effects of childhood poverty and chronic stress on emotion regulatory brain function in adulthood. *Proc. Natl. Acad. Sci.* **110**, 18442–18447 (2013).

27. Nusslock, R. & Miller, G. E. Early-Life Adversity and Physical and Emotional Health Across the Lifespan: A Neuroimmune Network Hypothesis. *Biol. Psychiatry* **80**, 23–32 (2016).

28. Duffy, K. A., McLaughlin, K. A. & Green, P. A. Early life adversity and health-risk behaviors: proposed psychological and neural mechanisms. *Ann. N. Y. Acad. Sci.* **1428**, 151–169 (2018).

29. Coles, J. A., Myburgh, E., Brewer, J. M. & McMenamin, P. G. Where are we? The anatomy of the murine cortical meninges revisited for intravital imaging, immunology, and clearance of waste from the brain. *Prog. Neurobiol.* **156**, 107–148 (2017).

30. Balin, B. J., Broadwell, R. D., Salcman, M. & El-Kalliny, M. Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey. *J. Comp. Neurol.* **251**, 260–280 (1986).

31. Herisson, F. *et al.* Direct vascular channels connect skull bone marrow and the brain surface enabling myeloid cell migration. *Nat. Neurosci.* **21**, 1209–1217 (2018).

32. Louveau, A. *et al.* Structural and functional features of central nervous system lymphatics. *Nature* **523**, 337–341 (2015).

33. Aspelund, A. *et al.* A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* **212**, 991–999 (2015).

34. Eide, P. K., Vatnehol, S. A. S., Emblem, K. E. & Ringstad, G. Magnetic resonance imaging provides evidence of glymphatic drainage from human brain to cervical lymph nodes. *Sci. Rep.* **8**, 7194 (2018).

35. Absinta, M. *et al.* Human and nonhuman primate meninges harbor lymphatic vessels that can be visualized noninvasively by MRI. *eLife* **6**, (2017).

36. Rua, R. & McGavern, D. B. Advances in Meningeal Immunity. *Trends Mol. Med.* **24**, 542–559 (2018).

37. González-de-Olano, D. & Álvarez-Twose, I. Mast Cells as Key Players in Allergy and Inflammation. *J. Investig. Allergol. Clin. Immunol.* **28**, 365–378 (2018).

38. Kunder, C. A., St John, A. L. & Abraham, S. N. Mast cell modulation of the vascular and lymphatic endothelium. *Blood* **118**, 5383–5393 (2011).

39. Forsythe, P. Mast Cells in Neuroimmune Interactions. *Trends Neurosci.* **42**, 43–55 (2019).

40. Skaper, S. D., Facci, L., Zusso, M. & Giusti, P. Neuroinflammation, Mast Cells, and Glia: Dangerous Liaisons. *Neurosci. Rev. J. Bringing Neurobiol. Neurol. Psychiatry* **23**, 478–498 (2017).

41. Dong, H. *et al.* Stabilization of Brain Mast Cells Alleviates LPS-Induced Neuroinflammation by Inhibiting Microglia Activation. *Front. Cell. Neurosci.* **13**, 191 (2019).

42. Sayed, B. A., Christy, A. L., Walker, M. E. & Brown, M. A. Meningeal Mast Cells Affect Early T Cell Central Nervous System Infiltration and Blood-Brain Barrier Integrity through TNF: A Role for Neutrophil Recruitment? *J. Immunol.* **184**, 6891–6900 (2010).

43. Zhuang, X., Silverman, A. J. & Silver, R. Brain mast cell degranulation regulates blood-brain barrier. *J. Neurobiol.* **31**, 393–403 (1996).

44. Russi, A. E., Walker-Caulfield, M. E. & Brown, M. A. Mast cell inflammasome activity in the meninges regulates EAE disease severity. *Clin. Immunol.* **189**, 14–22 (2018).

45. Arac, A. *et al.* Evidence that meningeal mast cells can worsen stroke pathology in mice. *Am. J. Pathol.* **184**, 2493–2504 (2014).

46. Levy, D., Burstein, R., Kainz, V., Jakubowski, M. & Strassman, A. M. Mast cell degranulation activates a pain pathway underlying migraine headache. *PAIN* **130**, 166–176 (2007).

47. Ayyadurai, S. *et al.* Frontline Science: Corticotropin-releasing factor receptor subtype 1 is a critical modulator of mast cell degranulation and stress-induced pathophysiology. *J. Leukoc. Biol.* **102**, 1299–1312 (2017).

48. D'Costa, S. *et al.* Mast cell corticotropin-releasing factor subtype 2 suppresses mast cell degranulation and limits the severity of anaphylaxis and stress-induced intestinal permeability. *J. Allergy Clin. Immunol.* **0**, (2018).

49. Theoharides, T. C. *et al.* Stress-induced intracranial mast cell degranulation: a corticotropin-releasing hormone-mediated effect. *Endocrinology* **136**, 5745–5750 (1995).

50. Pohl, C. S. *et al.* Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. *Neurogastroenterol. Motil. Off. J. Eur. Gastrointest. Motil. Soc.* **29**, (2017).

51. Baumeister, D., Akhtar, R., Ciufolini, S., Pariante, C. M. & Mondelli, V. Childhood trauma and adulthood inflammation: a meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-α. *Mol. Psychiatry* **21**, 642–649 (2016).

52. Kuhlman, K. R., Horn, S. R., Chiang, J. J. & Bower, J. E. Early life adversity exposure and circulating markers of inflammation in children and adolescents: A systematic review and meta-analysis. *Brain. Behav. Immun.* **86**, 30–42 (2020).

53. Antila, S. *et al.* Development and plasticity of meningeal lymphatic vessels. *J. Exp. Med.* **214**, 3645–3667 (2017).

54. Song, E. *et al.* VEGF-C-driven lymphatic drainage enables brain tumor immunosurveillance. *Nature* **577**, 689–694 (2020).

55. Koroleva, K. *et al.* Meningeal Mast Cells Contribute to ATP-Induced Nociceptive Firing in Trigeminal Nerve Terminals: Direct and Indirect Purinergic Mechanisms Triggering Migraine Pain. *Front. Cell. Neurosci.* **13**, (2019).

56. Nurkhametova, D. F. *et al.* Mast Cell Mediators as Pain Triggers in Migraine: Comparison of Histamine and Serotonin in the Activation of Primary Afferents in the Meninges in Rats. *Neurosci. Behav. Physiol.* **50**, 900–906 (2020).

57. Galli, S. J. *et al.* Approaches for Analyzing the Roles of Mast Cells and Their Proteases In Vivo. *Adv. Immunol.* **126**, 45–127 (2015).

58. Amini-Khoei, H. *et al.* On the role of corticosterone in behavioral disorders, microbiota composition alteration and neuroimmune response in adult male mice subjected to maternal separation stress. *Int. Immunopharmacol.* **66**, 242–250 (2019).

59. De Miguel, C., Obi, I. E., Ho, D. H., Loria, A. S. & Pollock, J. S. Early life stress induces immune priming in kidneys of adult male rats. *APSselect* **4**, F343–F355 (2017).

60. Avitsur, R., Hunzeker, J. & Sheridan, J. F. Role of early stress in the individual differences in host response to viral infection. *Brain. Behav. Immun.* **20**, 339–348 (2006).

61. Wang, Q. *et al.* Adolescent escitalopram prevents the effects of maternal separation on depression- and anxiety-like behaviours and regulates the levels of inflammatory cytokines in adult male mice. *Int. J. Dev. Neurosci.* **62**, 37–45 (2017).

62. Amini-Khoei, H. *et al.* Oxytocin mitigated the depressive-like behaviors of maternal separation stress through modulating mitochondrial function and neuroinflammation. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **76**, 169–178 (2017).

63. Ganguly, P., Honeycutt, J. A., Rowe, J. R., Demaestri, C. & Brenhouse, H. C. Effects of early life stress on cocaine conditioning and AMPA receptor composition are sex-specific and driven by TNF. *Brain. Behav. Immun.* **78**, 41–51 (2019).

64. Viola, T. W. *et al.* Acute neuroinflammation elicited by TLR-3 systemic activation combined with early life stress induces working memory impairments in male adolescent mice. *Behav. Brain Res.* **376**, 112221 (2019).

65. Estienne, M. *et al.* Maternal deprivation alters epithelial secretory cell lineages in rat duodenum: role of CRF-related peptides. *Gut* **59**, 744–751 (2010).

66. Li, B. *et al.* Endoplasmic reticulum stress is involved in the colonic epithelium damage induced by maternal separation. *J. Pediatr. Surg.* **51**, 1001–1004 (2016).

67. Stephan, M., Straub, R. H., Breivik, T., Pabst, R. & von Hörsten, S. Postnatal maternal deprivation aggravates experimental autoimmune encephalomyelitis in adult Lewis rats: reversal by chronic imipramine treatment. *Int. J. Dev. Neurosci.* **20**, 125–132 (2002).

68. Kinkead, R. & Gulemetova, R. Neonatal maternal separation and neuroendocrine programming of the respiratory control system in rats. *Biol. Psychol.* **84**, 26–38 (2010).

69. Wigger, D. C. *et al.* Maternal Separation Induces Long-Term Alterations in the Cardiac Oxytocin Receptor and Cystathionine γ-Lyase Expression in Mice. *Oxid. Med. Cell. Longev.* **2020**, e4309605 (2020).

70. Banqueri, M., Méndez, M. & Arias, J. L. Behavioral effects in adolescence and early adulthood in two length models of maternal separation in male rats. *Behav. Brain Res.* **324**, 77–86 (2017).

71. Gracia-Rubio, I. *et al.* Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **65**, 104–117 (2016).

72. George, E. D., Bordner, K. A., Elwafi, H. M. & Simen, A. A. Maternal separation with early weaning: a novel mouse model of early life neglect. *BMC Neurosci.* **11**, 123 (2010).

73. Brancato, A. *et al.* Sub-chronic variable stress induces sex-specific effects on glutamatergic synapses in the nucleus accumbens. *Neuroscience* **350**, 180–189 (2017).

74. Williams, E. S. *et al.* Androgen-Dependent Excitability of Mouse Ventral Hippocampal Afferents to Nucleus Accumbens Underlies Sex-Specific Susceptibility to Stress. *Biol. Psychiatry* (2019) doi:10.1016/j.biopsych.2019.08.006.

75. Peña, C. J. *et al.* Early life stress alters transcriptomic patterning across reward circuitry in male and female mice. *Nat. Commun.* **10**, (2019).

76. Baba, A. *et al.* Anti-Allergic Drugs Tranilast and Ketotifen Dose-Dependently Exert Mast Cell-Stabilizing Properties. *Cell. Physiol. Biochem.* **38**, 15–27 (2016).

77. Finn, D. F. & Walsh, J. J. Twenty-first century mast cell stabilizers. *Br. J. Pharmacol.* **170**, 23–37 (2013).

78. Tashiro, M. *et al.* Brain histamine H1 receptor occupancy of orally administered antihistamines measured by positron emission tomography with 11C-doxepin in a placebo-controlled crossover study design in healthy subjects: a comparison of olopatadine and ketotifen. *Br. J. Clin. Pharmacol.* **61**, 16–26 (2006).

79. Barnes, N. M., Costall, B., Kelly, M. E., Onaivi, E. S. & Naylor, R. J. Ketotifen and its analogues reduce aversive responding in the rodent. *Pharmacol. Biochem. Behav.* **37**, 785–793 (1990).

80. Lenz, K. M. *et al.* Mast Cells in the Developing Brain Determine Adult Sexual Behavior. *J. Neurosci.* **38**, 8044–8059 (2018).

81. Burks, C. A. *et al.* Ketotifen Modulates Mast Cell Chemotaxis to Kit-Ligand, but Does Not Impact Mast Cell Numbers, Degranulation, or Tumor Behavior in Neurofibromas of Nf1-Deficient Mice. *Mol. Cancer Ther.* **18**, 2321–2330 (2019).

82. Greenberg, G. & Burnstock, G. A novel cell-to-cell interaction between mast cells and other cell types. *Exp. Cell Res.* **147**, 1–13 (1983).
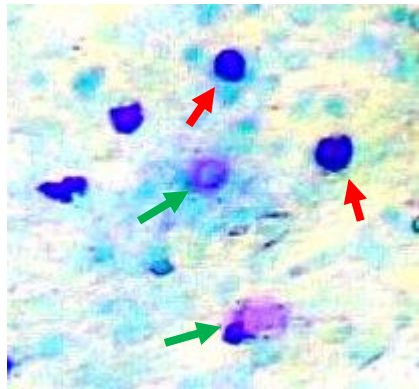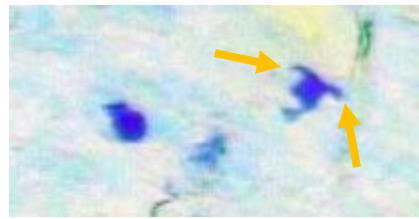
83. Rovere, F. D., Granata, A., Monaco, M. & Basile, G. Phagocytosis of Cancer Cells by Mast Cells in Breast Cancer. *Anticancer Res.* **29**, 3157–3161 (2009).

84. Walsh, D. R. *et al.* Regional mechanical and biochemical properties of the porcine cortical meninges. *Acta Biomater.* **80**, 237–246 (2018).

85. Andres, K. H., von Düring, M., Muszynski, K. & Schmidt, R. F. Nerve fibres and their terminals of the dura mater encephali of the rat. *Anat. Embryol. (Berl.)* **175**, 289–301 (1987).

86. Noseda, R., Melo-Carrillo, A., Nir, R.-R., Strassman, A. M. & Burstein, R. Non-Trigeminal Nociceptive Innervation of the Posterior Dura: Implications to Occipital Headache. *J. Neurosci. Off. J. Soc. Neurosci.* **39**, 1867–1880 (2019).

87. Rustenhoven, J. *et al.* Functional characterization of the dural sinuses as a neuroimmune interface. *Cell* **184**, 1000-1016.e27 (2021).

88. Allen, G., Buxton, R. B., Wong, E. C. & Courchesne, E. Attentional Activation of the Cerebellum Independent of Motor Involvement. *Science* **275**, 1940–1943 (1997).

89. Schmahmann, J. D. The role of the cerebellum in affect and psychosis. *J. Neurolinguistics* **13**, 189–214 (2000).

90. Schmahmann, J. D. Dysmetria of thought: clinical consequences of cerebellar dysfunction on cognition and affect. *Trends Cogn. Sci.* **2**, 362–371 (1998).

91. Teicher, M. H. *et al.* The neurobiological consequences of early stress and childhood maltreatment. *Neurosci. Biobehav. Rev.* **27**, 33–44 (2003).

92. Bauer, P. M., Hanson, J. L., Pierson, R. K., Davidson, R. J. & Pollak, S. D. Cerebellar volume and cognitive functioning in children who experienced early deprivation. *Biol. Psychiatry* **66**, 1100–1106 (2009).

93. Brooks, S. J. *et al.* Early-life adversity and orbitofrontal and cerebellar volumes in adults with obsessive–compulsive disorder: Voxel-based morphometry study. *Br. J. Psychiatry* **208**, 34–41 (2016).

94. Gutiérrez-Menéndez, A., Banqueri, M., Méndez, M. & Arias, J. L. How Does Maternal Separation Affect the Cerebellum? Assessment of the Oxidative Metabolic Activity and Expression of the c-Fos Protein in Male and Female Rats. *The Cerebellum* **19**, 68–77 (2020).

95. Zhu, Y., Yu, T. & Rao, Y. Temporal regulation of cerebellar EGL migration through a switch in cellular responsiveness to the meninges. *Dev. Biol.* **267**, 153–164 (2004).

96. Zhu, Y. *et al.* Role of the chemokine SDF-1 as the meningeal attractant for embryonic cerebellar neurons. *Nat. Neurosci.* **5**, 719–720 (2002).

97. Wilhelm, M., Silver, R. & Silverman, A. J. Central nervous system neurons acquire mast cell products via transgranulation. *Eur. J. Neurosci.* **22**, 2238–2248 (2005).

98. Louveau, A. *et al.* CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. *Nat. Neurosci.* **21**, 1380–1391 (2018).

99. Hu, X. *et al.* Meningeal lymphatic vessels regulate brain tumor drainage and immunity. *Cell Res.* **30**, 229–243 (2020).

100. Breslin, J. W. *et al.* Vascular endothelial growth factor-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism. *Am. J. Physiol.-Heart Circ. Physiol.* **293**, H709–H718 (2007).

101. Pal, S., Nath, S., Meininger, C. J. & Gashev, A. A. Emerging Roles of Mast Cells in the Regulation of Lymphatic Immuno-Physiology. *Front. Immunol.* **11**, (2020).

102. Bulfone-Paus, S. & Bahri, R. Mast Cells as Regulators of T Cell Responses. *Front. Immunol.* **6**, (2015).

103. Nakae, S. *et al.* Mast cells enhance T cell activation: Importance of mast cell-derived TNF. *Proc. Natl. Acad. Sci.* **102**, 6467–6472 (2005).

104. Russi, A. E., Walker-Caulfield, M. E., Guo, Y., Lucchinetti, C. F. & Brown, M. A. Meningeal mast cell-T cell crosstalk regulates T cell encephalitogenicity. *J. Autoimmun.* **73**, 100–110 (2016).

105. Klein, S. L. & Flanagan, K. L. Sex differences in immune responses. *Nat. Rev. Immunol.* **16**, 626–638 (2016).

106. Casimir, G. J., Lefèvre, N., Corazza, F. & Duchateau, J. Sex and inflammation in respiratory diseases: a clinical viewpoint. *Biol. Sex Differ.* **4**, 16 (2013).

107. Lasselin, J., Lekander, M., Axelsson, J. & Karshikoff, B. Sex differences in how inflammation affects behavior: What we can learn from experimental inflammatory models in humans. *Front. Neuroendocrinol.* **50**, 91–106 (2018).

108. Gater, R. *et al.* Sex Differences in the Prevalence and Detection of Depressive and Anxiety Disorders in General Health Care Settings: Report From the World Health Organization Collaborative Study on Psychological Problems in General Health Care. *Arch. Gen. Psychiatry* **55**, 405–413 (1998).

109. Vetvik, K. G. & MacGregor, E. A. Sex differences in the epidemiology, clinical features, and pathophysiology of migraine. *Lancet Neurol.* **16**, 76–87 (2017).

110. Tietjen, G. E. & Peterlin, B. L. Childhood Abuse and Migraine: Epidemiology, Sex Differences, and Potential Mechanisms. *Headache J. Head Face Pain* **51**, 869–879 (2011).

111. Dunn, S. E., Gunde, E. & Lee, H. Sex-Based Differences in Multiple Sclerosis (MS): Part II: Rising Incidence of Multiple Sclerosis in Women and the Vulnerability of Men to Progression of this Disease. *Curr. Top. Behav. Neurosci.* **26**, 57–86 (2015).

112. Mackey, E. *et al.* Sexual dimorphism in the mast cell transcriptome and the pathophysiological responses to immunological and psychological stress. *Biol. Sex Differ.* **7**, 60 (2016).

113. Mackey, E. *et al.* Perinatal androgens organize sex differences in mast cells and attenuate anaphylaxis severity into adulthood. *Proc. Natl. Acad. Sci.* **117**, 23751–23761 (2020).

114.   McClain, J. L. *et al.* Histamine-dependent interactions between mast cells, glia, and neurons are altered following early-life adversity in mice and humans. *Am. J. Physiol. Gastrointest. Liver Physiol.* **319**, G655–G668 (2020).

Table 1. Classification of duramater mast cells

Type	Description	Examples
Inactive (red arrows)	Mast cell is densely packed, most of the cytoplasm is dark purple, and the cell surface can be clearly delimited	
Active (green arrows)	Mast cell is not densely packed, the cytoplasm purple stain is scattered, and the cell surface is blurry	
Pseudopodia (yellow arrows)	One or more cytoplasmic extensions arise from the surface of the cell.	

A Early life treatment
PN days 0-17/28

Ketotifen + Adult Stress
PN day 56-58

Duramater collection
PN day 62

Males   Females

B CMA1

C IL-6

D Hdc

E TNF alpha

F TPh1

G VEGF-C

A Early life treatment
PN days 0-17/28

Adult Stress
PN day 56-58

Duramater collection
PN day 62

Males   Females

B CMA1

C IL-6

D Hdc

E TNF alpha

F TPh1

G VEGF-C

A Early life treatment
PN days 0-17/28

Undisturbed

Duramater collection
PN day 62

Skull Bone

Duramater
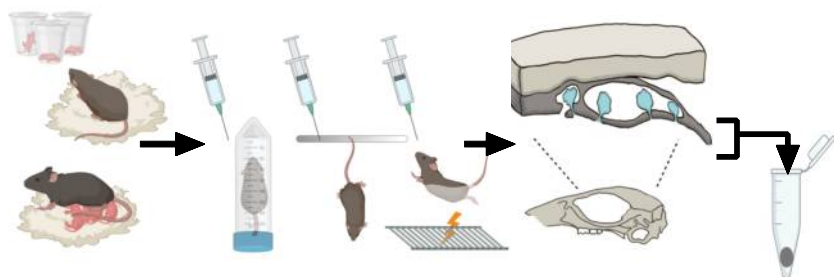
Males   Females

B CMA1

Normalized expression

**

NH   NMSEW
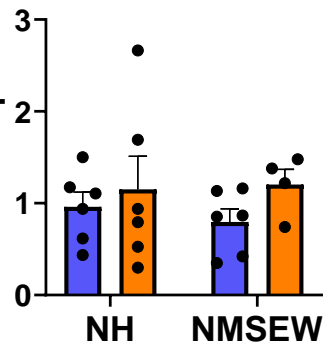
C IL-6

Normalized expression

****

NH   NMSEW
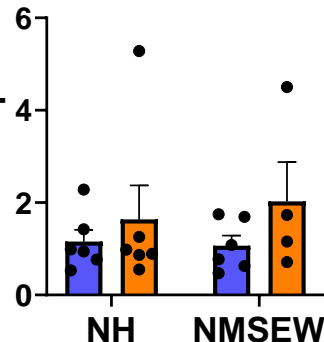
D Hdc

Normalized expression

NH   NMSEW
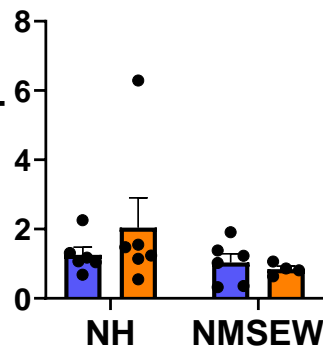
E TNF alpha

Normalized expression

*

NH   NMSEW
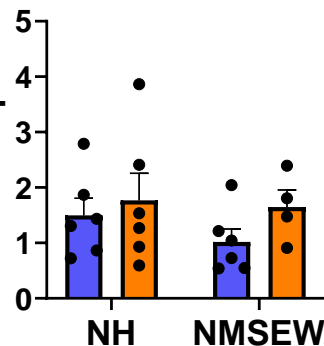
F Tph1

Normalized expression

NH   NMSEW

G VEGF-C

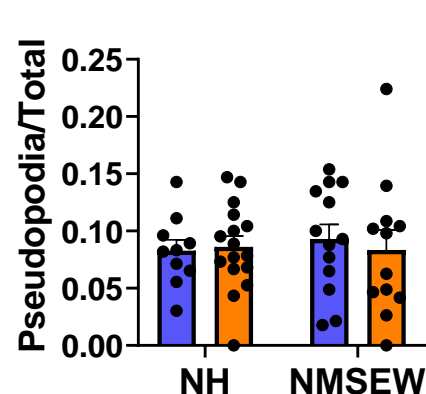Normalized expression

*

NH   NMSEW
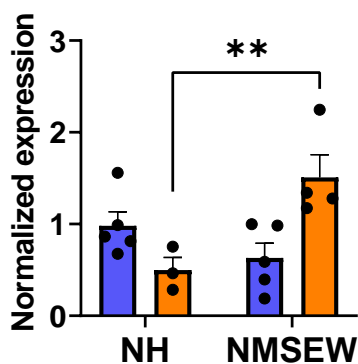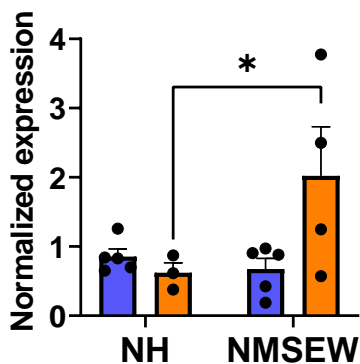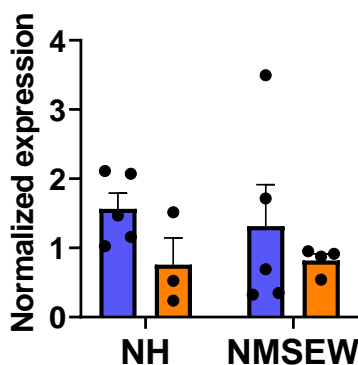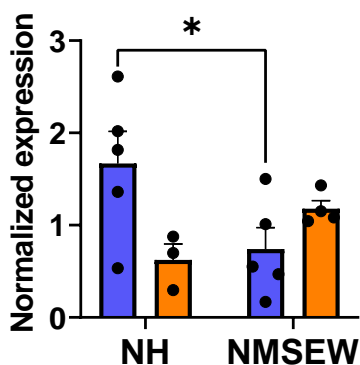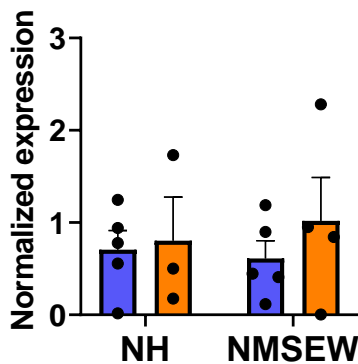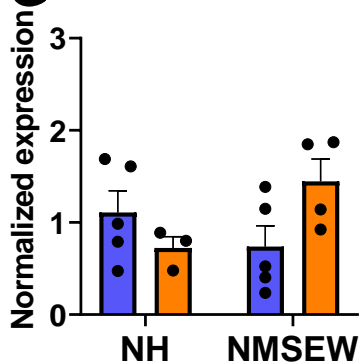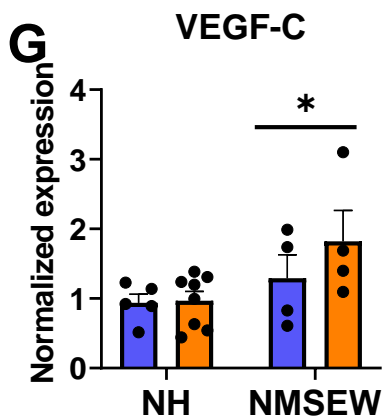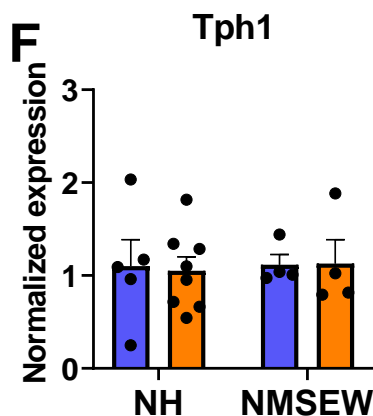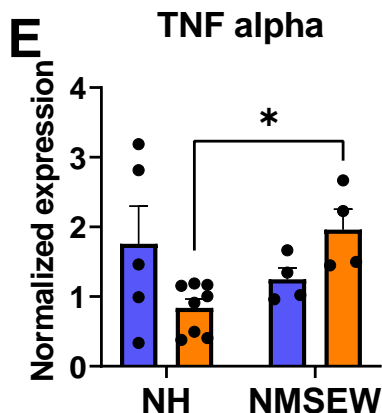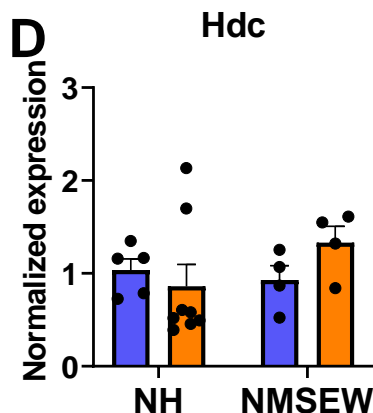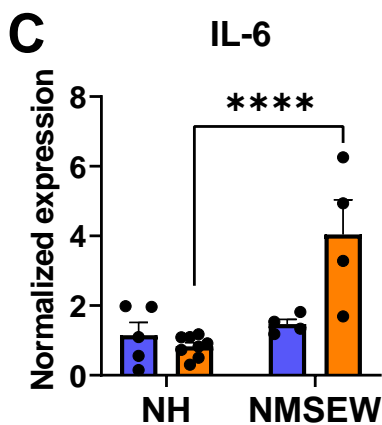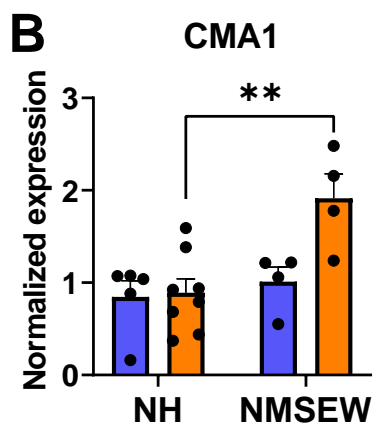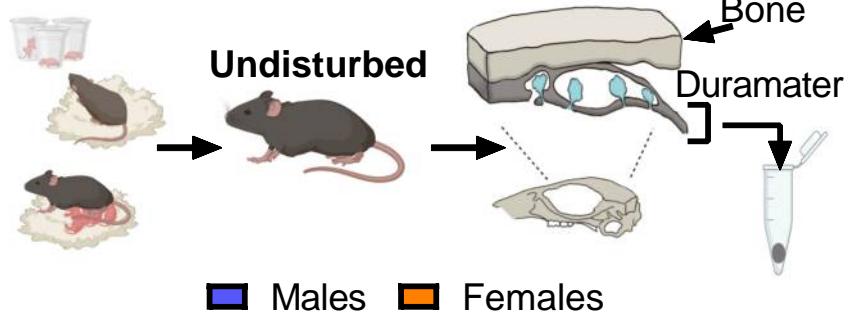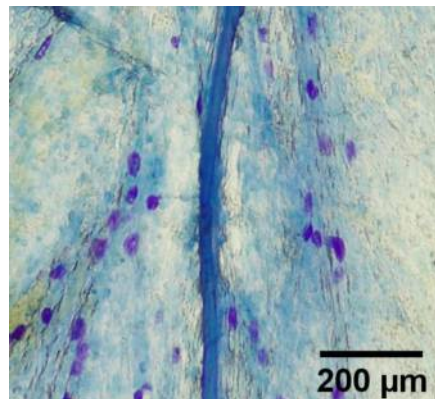
A SAGITTAL SINUS

B Mast cell number
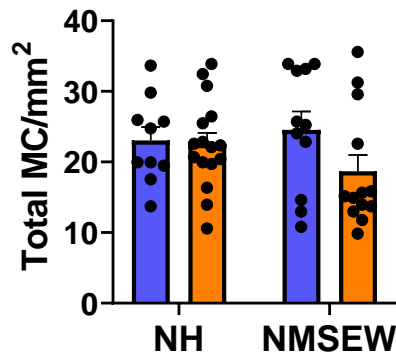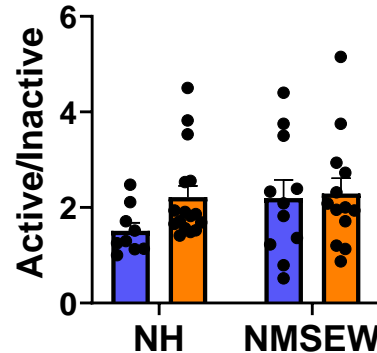
C Activation level

D Relative pseudopodia expression

E TRANSVERSE SINUS

F Mast cell number

G Activation level

H
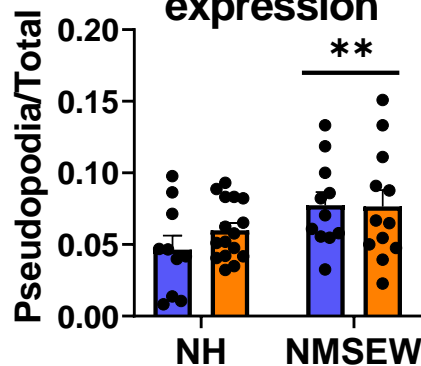
Males   Females
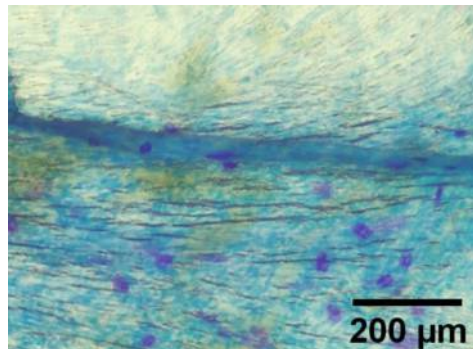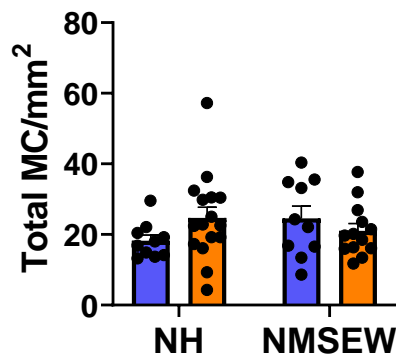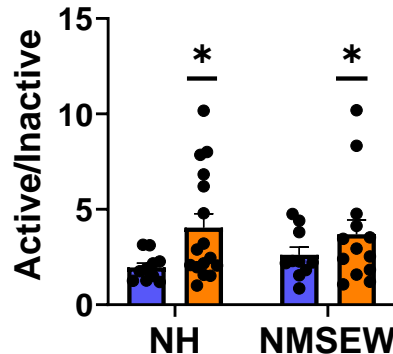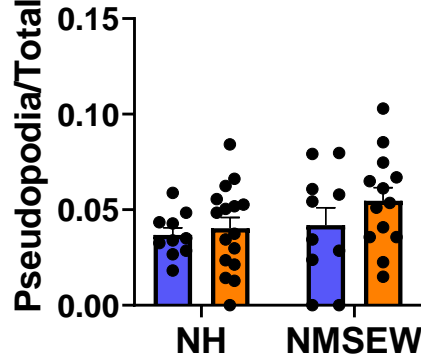
A Early life treatment
PN days 0-17/28

Undisturbed

Adult Stress
PN days 56-59

Tissue collection
PN day 62

SAGITTAL SINUS

PARIETAL PARIETAL

TRANSVERSE SINUS

INTER PARIETAL

B
Inactive
Pseudopodia
Active
100 μm

C INTERPARIETAL
200 μm

D Mast cell number
Total MC/mm²
NH   NMSEW   *

E Activation level
Active/Inactive
NH   NMSEW

F Relative pseudopodia expression
Pseudopodia/Total
NH   NMSEW   **

Males   Females

G PARIETAL
200 μm

H
Total MC/mm²
NH   NMSEW

I
Active/Inactive
NH   NMSEW   *

J
Pseudopodia/Total
NH   NMSEW

A

Sagittal

Parietal

Transverse

Interparietal

B Mast cell number

C Activation level

D Pseudopodia
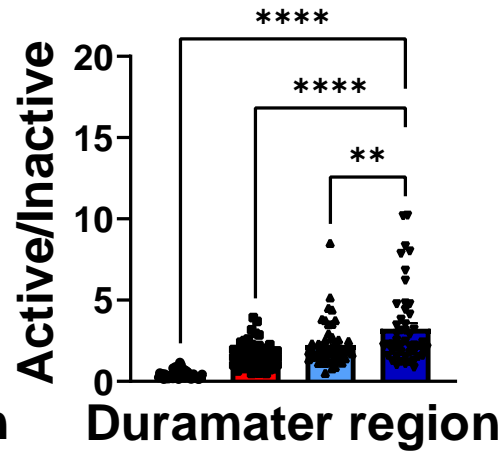
□ Parietal ■ Interparietal □ Sagittal Sinus ■ Transverse Sinus